Ocular biomarkers for Alzheimer's disease

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Chapter 1

Retinal and cerebral microvasculopathy: relationships and their genetic contributions

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Abstract

Purpose: Retinal microvasculopathy may reflect small vessel disease in the brain. Here we test the relationships between retinal vascular parameters and small vessel disease, the influence of cardiovascular risk factors on these relationships, and their common genetic background in a monozygotic twin cohort.

Methods: We selected 134 cognitively healthy individuals (67 monozygotic twin pairs) aged ≥60 from the Netherlands Twin Register for the EMIF-AD PreclinAD study. We measured 7 retinal vascular parameters averaged over both eyes using fundus images analyzed with Singapore I Vessel Assessment. Small vessel disease was assessed on MRI by a volumetric measurement of periventricular and deep white matter hyperintensities. We calculated associations between RVPs and WMH, estimated intra-twin pair correlations, and performed twin specific analyses on relationships of interest.

Results: Deep white matter hyperintensities volume was positively associated with retinal tortuosity in veins ($p=0.004$) and fractal dimension in arteries ($p=0.001$) and veins ($p=0.032$), periventricular white matter hyperintensities volume was positively associated with retinal venous width (CRVE, $p=0.028$). Intra-twin pair correlations were moderate to high for all small vessel disease/retinal vascular parameter variables ($r=0.49$-0.87, $p<0.001$). Cross-twin cross-trait analyses showed that retinal venous tortuosity of twin 1 could predict deep white matter hyperintensities volume of the co-twin ($r=0.23$, $p=0.030$). Within twin-pair differences for retinal venous tortuosity were associated with within twin-pair differences in deep white matter hyperintensities volume ($r=0.39$, $p=0.001$).

Conclusion: Retinal arterial fractal dimension and venous tortuosity have associations with deep white matter hyperintensities volume. Twin specific analyses suggest that retinal venous tortuosity and deep white matter hyperintensities volume have a common etiology driven by both shared genetic factors and unique environmental factors, supporting the robustness of this relationship.

Introduction

The eye, and especially the retina, may reflect vascular pathology elsewhere in the body, and is easily assessable through direct imaging like fundus photography. Subtle changes of the microvasculature of the retina, such as retinal vessel diameter changes or increased tortuosity, can be measured using automated detection software on fundus photographs (1).

The retina shares many similarities with the brain, being embryologically derived from the same tissue and possessing similar structural and functional features such as a blood-retina barrier and glial cell connections (2). This makes the eye an ideal potential biomarker for vascular and degenerative processes occurring in the brain (2-6). Many degenerative diseases of the brain also contain a vascular component. Vascular dementia is an obvious example, but also in diseases such as Alzheimer's disease vascular changes play an important role (7, 8). These pathological vascular changes of the brain are summarized under the term cerebral small vessel disease, such as white matter hyperintensities on MRI (9-11). White matter hyperintensities are commonly observed white spots in the cerebral white matter of especially elderly people on MRI, and are thought to reflect (micro)vascular damage (12). Several groups have found relationships between retinal vascular parameters and cerebral small vessel disease, especially in people predisposed to cerebral small vessel disease (13-18).

Vascular changes in the eye and the brain show a moderate to high level of genetic influence (19). Heritability (variation explained by the genome) estimates lie between 0.49-0.83 for ophthalmological vascular parameters (20-23) and between 0.64-0.77 for small vessel disease (24-27). By performing a study on monozygotic twins, we aimed to elucidate the genetic and environmental influence on possible relationships between these two vascular systems. For this purpose, two sets of analyses will be performed; a cross-twin cross trait and a twin-difference analysis. A cross-twin cross-trait analysis takes the trait of twin 1 to predict a different trait of the co-twin. If a relationship is found, this suggests that the relationship between said traits is at least partially explained by shared factors, either genetic or environmental, between the twins (28). A twin-difference analysis correlates the within twin-pair differences of a continuous trait to the within twin-pair differences in a second trait. If a relationship between these differences is found, this suggests a unique environmental factor mediating both traits, as differences within monozygotic twins can only be due to environmental exposures that are not shared by twins. If there is a causal relationship between the two traits and the causal trait is partly genetic, and partly environmental, difference scores will also be correlated (29).

A confounding factor in the relationship between vasculature in the eye and the brain is systemic disease, as systemic vascular disease shows relationships to both retinal vessel

parameters as well as brain small vessel disease. For example, hypertension has been shown to be associated with decreased arteriolar diameter (30-34) and a smaller arteriovenous ratio was found to be related to atherosclerotic findings (35, 36) and an increased chance of developing diabetes mellitus (37, 38). Additionally, it is known that cardiovascular risk factors such as hypertension play an important role in the development of cerebral small vessel disease (39, 40).

In this study, we aimed to 1) test the associations between retinal vascular parameters and small vessel disease in the context of cardiovascular risk factors, 2) estimate the upper limit of the genetic contribution to retinal vascular parameters and small vessel disease in a population of healthy, elderly, monozygotic twins and 3) investigate the underlying mechanism of the associations between retinal vascular parameters and small vessel disease with cross-twin cross-trait and within-pair difference analyses.

Methods

Participants

We invited 217 participants aged ≥60 from the Netherlands Twin Register (27, 41) who take part in the Amsterdam sub-study of the European Medical Information Framework for Alzheimer's Disease (EMIF-AD) PreclinAD cohort. The study followed the Tenets of the Declaration of Helsinki and written informed consent was obtained from all participants. The study was approved by the Medical Ethics Committee of the VU University Medical Center in Amsterdam.

Inclusion criteria were: monozygosity, age ≥60 years, cognitively healthy as defined by: Telephone Interview for Cognitive Status modified (TICS-m) score >22 (42), Geriatric Depression Scale (GDS) score <11 (43), Consortium to Establish a Registry for Alzheimer's Disease (CERAD) 10 word list immediate and delayed recall >-1.5 SD of age adjusted normative data (44) and Clinical Dementia Rating (CDR) scale of 0 with a score on the memory sub domain of 0 (45).

Exclusion criteria were: stroke resulting in physical impairment, neurodegenerative disorders, cancer with terminal life expectancy, uncontrolled diabetes mellitus, alcohol consumption >35 units (1 unit = 10ml or 8g of pure alcohol) per week.

Cardiovascular risk factors

Clinical data were collected during a face-to-face interview for medical history, medication intake, smoking habits, and educational attainment. All participants also underwent physical examinations. Blood pressure was measured 3 times in a lying position with a 5-minute interval between measurements, and the mean of these 3 measurements was used for the analysis. After a minimum 2-hour fasting period,

participants underwent a blood- draw to determine lipid profile and glycated hemoglobin in the morning. The cardiovascular risk profile for each participant was summarized using the Framingham Risk Score, which includes the following factors: age, gender, total cholesterol, high density lipoprotein (HDL), systolic blood pressure, antihypertensive medication use, diabetes mellitus, and smoking (46). This risk index represents the 10-year risk of a major cardiovascular event.

Ophthalmological examination

All participants underwent the following ophthalmological examinations: best corrected visual acuity, intra-ocular pressure, refraction data, slit lamp examination, indirect fundoscopy and fundus photography. Tropicamide 0.5% was used for pupil dilation to enable these examinations. All photographs were assessed by an experienced ophthalmologist (HTN) for unexpected pathology. Participants suffering from ophthalmological conditions interfering with retinal vasculature or image quality were excluded from analysis (cataract, macular degeneration, glaucoma, diabetic retinopathy, vascular occlusions), as well as ungradable images.

Fundus photography and quantitative assessment of retinal vasculature

Digital fundus images of 50° field of view were obtained from both eyes of most participants (Topcon TRC 50DX type IA; Topcon Medical Systems, Inc., Oakland, CA, USA), centered on the optic nerve head. These images were graded by a trained grader (JAvdK) using the Singapore I Vessel Assessment (SIVA) software (version 3.0, National University of Singapore, Singapore) (47-49). Values from both eyes of every participant were averaged, if only one suitable image was present only this eye was included. Intra-rater scores were calculated in 20 patients. Based on this, we selected 7 mostly dimensionless retinal vascular parameters to account for refraction error with an intra-observer intra-class correlation, absolute agreement, of >0.80 for the analyses. These are: central retinal artery equivalent, central retinal vein equivalent, arteriole–venular ratio, fractal dimension of the arteriolar network, fractal dimension of the venular network, curvature tortuosity of the arterioles and curvature tortuosity of the venules. All values for retinal vascular parameters were obtained within zone C (i.e. 0.5 – 2 disc diameters around the optic nerve head).

Magnetic Resonance Imaging (MRI)

Whole brain scans were obtained using a 3T scanner using an 8-channel head coil (Philips Ingenuity Time-of-Flight PET/MRI-scanner; Philips Medical Systems, Best, The Netherlands). Isotropic structural three-dimensional (3D) T1-weighted images (1.00 mm^3 isotropic voxels, repetition time (TR)=7.9 ms, echo time (TE)=4.5 ms, flip angle=8 degrees) and 3D sagittal FLAIR sequences (1.12 mm3 isotropic voxels, TR=4800 ms, TE=279 ms, inversion time=1650 ms) were acquired. An experienced neuroradiologist visually

assessed all scans for incidental findings. The scans were visually rated by a single experienced rater (MtK), who was blinded to twin pairing. Cerebral small vessel disease was assessed by white matter hyperintensities, using 3D sagittal FLAIR, on MRI. White matter hyperintensities were automatically segmented using a previously described algorithm (27, 50). In short, the algorithm can make a distinction between different types of abnormal image patterns without pathological a priori knowledge, enabling detection of abnormal intensity clusters, which is particularly useful in segmenting white matter hyperintensities. The white matter hyperintensities map was then dichotomized into periventricular and deep regions using the relative distance between the ventricular surface and the cortical sheet at a ratio of 50%/50%. Total intra-cranial volume measurements were obtained from the application of a label fusion algorithm (51).

Statistical analysis

To normalize the distribution for white matter hyperintensities volume and arterial and venular tortuosity, a Log transformation was applied. White matter hyperintensities were further corrected for head size using the total intracranial volume. Analyses with these as dependent variables are thus reported in ratios instead of regression coefficients. A ratio can take any value from 0 onwards, where 1 means there is no relationship, <1 means there is a negative relationship (i.e. with an increase in the independent variable, the dependent variable will decrease) and >1 means there is a positive relationship (i.e. with an increase in the independent variable, the dependent variable will also increase). Relationships between retinal vascular parameters, white matter hyperintensities and cardiovascular risk factors were investigated with Generalized Estimating Equations (GEE) using SPSS (version 22; IBM, Chicago, IL, USA), to correct for clustering in the data from twin pairs as well as confounders such as age and gender. Monozygotic siblings are very much alike and should thus not be treated as separate individuals, GEE is a type of regression analysis which can take such dependencies into account. Intra-twin pair correlations were calculated using Pearson/Spearman correlation coefficients. An intra-twin pair correlation is the correlation between the two siblings within a twin pair. One would thus expect this correlation to be high when a trait is mainly determined by shared factors, of either genetic or environmental etiology. Cross-twin cross-trait analyses, which use the trait of twin 1 to predict a different trait of the co-twin, were performed using Structural Equation Modelling implemented in OpenMx running in RStudio (version 1.1.383; RStudio, Inc., Boston, MA, USA). Twin difference analyses were performed by using SPSS. Cross-twin cross-trait and twin difference analyses were performed on significant relationships between retinal vascular parameters and white matter hyperintensities only. Analyses were corrected for age and gender, standardized values for retinal fractal dimension of the veins and retinal tortuosity of both veins and arteries were used for the cross-twin cross-trait analyses. Standardizing values means you make a Z-score out of a parameter (with a mean of 0 and a standard deviation of 1.96). We

applied a standardization to some parameters for this particular analysis, because the script used for this analysis has trouble in dealing with values of a very different size (in this case comparing very small values to values around 3).

Results

Of the 217 approached subjects, 15 were excluded due to: epileptic seizures (N=1), heart problems (N=1), not meeting neuropsychological inclusion criteria (N=3), neurodegenerative disease (N=2), conditions disabling a hospital study visit (N=4), transient ischemic attacks (N=1) or being a sibling to one of the excluded cases (N=3), leaving a total of 202 participants (100 monozygotic twin pairs and 2 singletons). Of these, 12 were excluded from analyses due to ophthalmological disease. Of the remaining 190 participants, only 134 participants (67 twin pairs) were complete pairs who had useable data for most included parameters (i.e. sufficient quality fundus pictures and MRI data, complete Framingham Risk Score). This subset was demographically very similar to the whole population. Table 1 shows the demographics of the population in this study.

After correction for clustering, age, gender and total intracranial volume, only increased central retinal vein equivalent was related to a higher volume of periventricular white matter hyperintensities (table 2). An increased fractal dimension of arteries and veins and increased tortuosity of veins were related to deep white matter hyperintensities volume. Table 2 shows all the associations between retinal vascular parameters and white matter hyperintensities volumes.

To look for possible mediating cardiovascular risk factors in the above described relationships, cardiovascular risk factors were analyzed in relation to both retinal vascular parameters and small vessel disease (supplementary table 1). None of the cardiovascular risk factors were significantly related to both the retinal vascular parameters and small vessel disease in any of the relationships described above. When we repeated the analyses of the associations between retinal vascular parameters and small vessel disease with correction for Framingham risk score, the associations remained similar (supplementary table 2).

The intra-twin pair correlations were moderate to high for all small vessel disease/retinal vascular parameters variables (table 3).

Table 1: demographics from study population

N	*134*
Age (years)	*69.9 (±7.8)*
Sex, female N (%)	*72 (53.7%)*
Best corrected visual acuity (LogMAR)	*0.02 (±0.10)*
Intra-ocular pressure (mmHg)	*14.2 (±2.4)*
Spherical Equivalent (both eyes averaged)	*0.27 (±1.9)*
Mini Mental State Exam	*29.0 (±1.2)*
Cardiovascular risk factors:	
- Hypertension, N (%)	*49 (36.6%)*
- Diabetes mellitus type 2, N (%)	*6 (4.5%)*
- Mean arterial pressure (mmHg)	*107.2 (±11.2)*
- Smoking, N (%)	*16 (11.9%)*
- Body mass index (kg/m^2)	*25.9 (±3.6)*
- Framingham risk score	*26.7 (±14.9)*
White matter hyperintensities (WMH):	
- Periventricular WMH volume (mm^3)	*3020 (±3891)*
- Deep WMH volume (mm^3)	*1832 (±3054)*
- Total intracranial volume (mm^3)	*1397626 (±125704)*
Retinal vascular parameters:	
- Central retinal artery equivalent	*127.5 (±11.8)*
- Central retinal vein equivalent	*196.8 (±19.0)*
- Arteriole–venular ratio	*0.651 (±0.054)*
- Fractal dimension of arteries	*1.176 (±0.047)*
- Fractal dimension of veins	*1.157 (±0.049)*
- Curvature tortuosity of arteries (*10^{-4})	*0.632 (±0.165)*
- Curvature tortuosity of veins (*10^{-4})	*0.640 (±0.155)*

Data are means unless otherwise specified.

For the associations found between the retinal vascular parameters and small vessel disease (table 2), we performed cross-twin cross-trait and twin difference analysis. Cross-twin cross-trait analysis showed a relationship between retinal tortuosity of the veins of twin 1 with deep white matter hyperintensities volume of the co-twin (r=0.23, p=0.030, table 4), while other markers showed a trend (p≈0.1, table 5).

Within twin-pair differences for retinal venous tortuosity were related to within twin-pair differences in deep white matter hyperintensities volume (r=0.39, p=0.001, table 5).

Table 2: Associations between retinal vascular parameters and white matter hyperintensities

	Periventricular white matter hyperintensities volume Ratio, P-value (95% CI of ratio)	**Deep white matter hyperintensities volume** Ratio, P-value (95% CI of ratio)
Central retinal artery equivalent*	*1.690, p=0.459 (0.421 to 6.792)*	*1.429, p=0.657 (0.296 to 6.918)*
Central retinal vein equivalent*	***2.799, p=0.028 (1.119 to 6.998)***	*1.888, p=0.274 (0.605 to 5.888)*
Arteriole-venular ratio	*0.129, p=0.167 (0.007 to 2.360)*	*0.256, p=0.438 (0.008 to 7.980)*
Fractal dimension of arteries	*1.622, p=0.774 (0.060 to 43.954)*	***317.687, p=0.001 (12.246 to 8241.381)***
Fractal dimension of veins	*4.529, p=0.393 (0.141 to 145.546)*	***36.224, p=0.032 (1.352 to 970.510)***
Curvature tortuosity of arteries†	*1.982, p=0.273 (0.583 to 6.730)*	*3.100, p=0.088 0.845 to 11.376*
Curvature tortuosity of veins†	*2.937, p=0.116 (0.765 to 11.267)*	***7.995, p=0.004 1.967 to 32.486***

Beta's are reported in ratios due to a log transformation applied to the dependent variables. **Bold** values are significant at $p<0.05$. Generalized estimating equations, corrected for age, gender and total intracranial volume. Brain parameters were chosen as the dependent variables. * = reported in steps of 100, † = reported in steps of 10^{-4}.

Table 3: intra-twin pair correlation coefficients.

	Correlation coefficient	**p-value**
Small vessel disease:		
- Periventricular white matter hyperintensities volume	*0.87*	*<0.001*
- Deep white matter hyperintensities volume	*0.84*	*<0.001*
Retinal vascular parameters:		
- Central retinal artery equivalent	*0.64*	*<0.001*
- Central retinal vein equivalent	*0.64*	*<0.001*
- Arteriole-venular ratio	*0.49*	*<0.001*
- Fractal dimension of arteries	*0.52*	*<0.001*
- Fractal dimension of veins	*0.64*	*<0.001*
- Curvature tortuosity of arteries	*0.71*	*<0.001*
- Curvature tortuosity of veins	*0.69*	*<0.001*

Table 4: Cross-twin cross-trait correlations

	Periventricular white matter hyperintensities volume* r (p-value)	**Deep white matter hyperintensities volume*** r (p-value)
Central retinal artery equivalent	*NA*	*NA*
Central retinal vein equivalent	*0.17 (p=0.098)*	*NA*
Arteriole-venular ratio	*NA*	*NA*
Fractal dimension of arteries	*NA*	*0.16 (p=0.101)*
Fractal dimension of veins†	*NA*	*0.17 (p=0.100)*
Curvature tortuosity of arteries*†	*NA*	*NA*
Curvature tortuosity of veins*†	*NA*	***0.23 (p=0.030)***

For significant relationships between retinal vascular parameters and small vessel disease. **Bold** values are significant at p<0.05. Dependent variables were corrected for age, gender and total intracranial volume. NA = not applicable. * = Log transformation applied. † = standardized values used.

Table 5: Twin difference correlations

	Periventricular white matter hyperintensities volume r (p-value)	**Deep white matter hyperintensities volume** r (p-value)
Central retinal artery equivalent	*NA*	*NA*
Central retinal vein equivalent	*0.20 (p=0.103)*	*NA*
Arteriole-venular ratio	*NA*	*NA*
Fractal dimension of arteries	*NA*	*0.11 (p=0.392)*
Fractal dimension of veins	*NA*	*0.13 (p=0.284)*
Curvature tortuosity of arteries	*NA*	*NA*
Curvature tortuosity of veins	*NA*	***0.39 (p=0.001)***

For significant relationships between retinal vascular parameters and small vessel disease. **Bold** values are significant at p<0.05. NA = not applicable. RVPs = Retinal Vascular Parameters, SVD = Small Vessel Disease.

Discussion

Increased tortuosity of retinal veins and increased fractal dimension of retinal arteries and veins were related to a higher volume of deep white matter hyperintensities. An increase in the width of retinal veins was associated with a higher volume of periventricular white matter hyperintensities. Retinal venous tortuosity was also associated to deep white matter hyperintensities in cross-twin cross-trait and twin difference analysis, suggesting that these vascular changes have a shared underlying factor related to genetic and non-shared environmental factors.

We found that retinal venous tortuosity was positively associated with deep white matter hyperintensities volume. In the retina, increased vascular tortuosity is thought to be indicative of vessel wall dysfunction, tissue hypoxia, disturbed blood-retina barrier and disturbed blood flow (52). Similar mechanisms have been described to lead to white matter hyperintensities in the brain (53), suggesting similar processes on a microvascular level are occurring in synchrony in both the eye and the brain. This was supported by cross-twin cross-trait and twin-difference analyses. Cross-twin cross-trait analyses showed that the retinal venous tortuosity of twin 1 was associated with deep white matter hyperintensities volume of the co-twin. This means that the relationship between these traits is partly explained by shared factors between the twins (28). It is likely that in our elderly cohort, ages 60 and over, the association is mainly explained by genetic factors, as discussed below. Twin difference analysis showed that within twin-pair differences for retinal venous tortuosity were related to within twin-pair differences in deep white matter hyperintensities volume. As any difference between monozygotic twins is due to environmental factors unique to each twin, this means that the association is also driven by non-shared environmental factors, possibly supporting a causal relationship between these traits (29). As a direct causal influence of one trait on the other is unlikely in our case, our results suggest there is a common unique environmental factor influencing both retinal venous tortuosity and deep white matter hyperintensities volume, in addition to shared genetic factors.

An increase in retinal venous tortuosity has been found to be associated with hypertension (49). Hypertension also has a relationship to white matter hyperintensities volume (54), so it would seem likely that the relationship between retinal venous tortuosity and white matter hyperintensities volume was driven through hypertension. Yet, a diagnosis of hypertension did not show a relationship to venous tortuosity in our study (supplementary table 1), and adding Framingham risk score to our model did not change the significance of the relationship found between venous tortuosity and deep white matter hyperintensities volume (supplementary table 2). This suggests there is a relationship between the tortuosity of veins in the retina and deep white matter hyperintensities volume regardless of cardiovascular risk factors such as hypertension.

Our finding that increased fractal dimension in the retina was associated with higher volume of deep white matter hyperintensities was unexpected. Reduced fractal dimension has been associated with stroke, cognitive dysfunction and hypertension (3, 48, 55-59), and is thought to represent microvascular damage, due to destruction and collapse of small vessels, thus creating a 'simpler' network (60). As white matter hyperintensities also represents vascular damage (53), one would expect that decreased retinal fractal dimension is associated with higher white matter hyperintensities volume, rather than the opposite. An explanation could be a physiological variation in the fractal dimension of the retinal vasculature. It may be that those who are born with a more

'complex' network of retinal vessels may have a different make-up of vessels in the brain as well, which makes individuals more vulnerable to the development of white matter hyperintensities. Cross-twin cross-trait and twin difference analysis did not show any associations, suggesting that the association between fractal dimension and deep white matter hyperintensities does not have a shared etiology.

We found that increased venous width (central retinal vein equivalent) was associated with increased periventricular white matter hyperintensities volume. Other studies have shown that increased central retinal vein equivalent was related to cardiovascular risk factors such as hypertension, diabetes and smoking behavior (31, 61, 62). It is postulated that widening of the venules may reflect endothelial dysfunction, and it is hypothesized to be a general marker of retinal ischemia and hypoperfusion (62). Our results are in line with this; white matter hyperintensities represent (micro)vascular damage in the brain, and an increase in central retinal vein equivalent in the eye could suggest a similar process occurring in the eye. Cross-twin cross-trait and twin difference analysis did not show any associations, suggesting that the association between retinal venous width and deep white matter hyperintensities does not have a shared etiology.

Interestingly, most relationships between the eye and brain in this study were found with deep white matter hyperintensities, rather than periventricular white matter hyperintensities. Periventricular white matter hyperintensities has been shown to have a stronger association with cardiovascular risk factors, as it is supposed to be particularly vulnerable to decreases in blood flow due to it being anatomically located at the arterial border zone (63). This was confirmed by our study; after correction for multiple confounders, only periventricular white matter hyperintensities showed significant relationships with smoking behavior and Framingham risk score (supplemental table 1). As both periventricular white matter hyperintensities and microvascular changes of the eye are associated with cardiovascular risk factors , one would expect the relationship between microvascular changes in the eye to be more strongly related to periventricular white matter hyperintensities rather than deep white matter hyperintensities. This suggests that the underlying pathophysiology of retinal vascular parameters is likely to be heterogeneous.

For small vessel disease, we found high intra-twin pair correlations ranging from 0.84-0.87. Other studies have found similar contributions of genes to these parameters, with intra twin pair correlations ranging from 0.74-0.77 in monozygotic twins, corresponding to estimated heritabilities ranging from 0.64-0.77 for white matter hyperintensities lesions (25). For retinal vascular parameters, we found intra twin pair correlations ranged from 0.49-0.71. This matches what was found by other studies, as estimated intra twin pair correlations of 0.51-0.88, with corresponding heritabilities ranging from 0.57-0.83, are reported (20-22, 64).

It was a strength of the study that all participants were very well characterized, in both neurological and ophthalmological sense. Extensive screening was performed before inclusion of the participants for analysis. Another strength of the study was that we included the role of cardiovascular risk factors in the relationships between the eye and brain. Most other studies on this topic look at only the relationship between eye-brain, eye-systemic or brain-systemic. In this study, we also looked at the relationship between retinal vascular parameters and small vessel disease when corrected for cardiovascular disease.

We did not include dizygotic twins and therefore could not estimate the contribution of genetic factors and shared environmental factors to the twin associations. In older twins however, the estimated contribution of shared environmental effects is lower, as these twins have spent most of their life apart. This is also the case for both white matter hyperintensities volume and retinal vascular parameters, as other studies have shown very low contributions of the shared environment on these parameters (20, 21, 25).

We did not correct for multiple testing, as the nature of this study was mostly explorative. Still, several of the relationships found in this study (venous tortuosity and deep white matter hyperintensities volume, fractal dimension and deep white matter hyperintensities volume) were of such significance that they would survive a correction for multiple testing, suggesting a quite robust relationship.

It is very possible, and even highly likely, that other systemic factors/cardiovascular risk factors we did not include in our analyses are responsible for causing the relations found within this study. The relations found are very unlikely to be of a causative nature, i.e. vascular changes in the eye do not cause changes in white matter hyperintensities volume in the brain. It is thus likely that a common factor causes changes in both these domains, explaining why a relation between the two is found. We have tried to elucidate what factor this could be by looking at some of the more obvious systemic parameters. There are however, infinitely more (systemic) factors affecting the vascular state of the human body. It is impossible to look at all cardiovascular risk factors known thus far, so we limited our search to the more common ones (mostly the ones also used to compile the Framingham risk score). Additionally, our ultimate goal behind this study is to develop an easy and non-invasive biomarker to gain more insight into the vascular state of the brain. Regardless of what causes the relation between the eye and brain parameters within this study, if these eye parameters can be used to obtain information we would otherwise have to use an MRI for, we have already gained an invaluable biomarker.

In conclusion, this study showed that there was a moderate to high correlation between monozygotic twins for retinal vascular parameters and cerebral small vessel disease, suggesting a relatively big contribution of genes to these parameters. Furthermore, both fractal dimension of arteries and veins as well as tortuosity of veins in the retina were

related to deep white matter hyperintensities, and retinal venous width was related to periventricular white matter hyperintensities. The relationship between venous tortuosity and deep white matter hyperintensities volume was also significant on a cross-twin cross-trait as well as a twin difference analysis, giving us more insight into the nature of this relationship, but also strongly supporting its robustness. This relationship in particular may be of interest to explore further, to elucidate whether retinal venous tortuosity may have diagnostic properties in establishing those at risk of small vessel disease, as obtaining information using the eye can be a considerably more patient friendly and cheaper alternative to MRI scanning.

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Supplementary table 1: Associations between retinal vascular parameters/white matter hyperintensities and cardiovascular risk factors.

	CRAE	**CRVE**	**AVR**	**FDa**	**FDv**	**cTORTa**	**cTORTv**	**pWMH volume**	**dWMH volume**
	Beta p-value (95% CI)	Beta p-value (95% CI)	Beta p-value (95% CI)	Beta p-value (95% CI)	Beta p-value (95% CI)	Ratio p-value (95% CI)	Ratio p-value (95% CI)	Ratio p-value (95% CI)	Ratio p-value (95% CI)
Diabetes Mellitus type II	***-9.086*** ***p<0.001*** ***(-13.058 to -5.114)***	***-25.901*** ***p=0.022*** ***(-48.072 to -3.729)***	*0.027* *p=0.195* *(-0.014 to 0.067)*	*0.646** *p=0.833* *(-5.370 to 6.661)*	***1.831**** ***p=0.046*** ***(0.0315 to 3.631)***	*0.975†* *p=0.967* *(0.293 to 3.243)*	*0.698†* *p=0.521* *(0.233 to 2.092)*	*1.050* *p=0.837* *(0.661 to 1.671)*	*0.615* *p=0.274* *(0.257 to 1.469)*
Hypertension	*3.348* *p=0.122* *(-0.892 to 7.588)*	***5.094*** ***p=0.044*** ***(0.135 to 10.054)***	*0.164** *p=0.864* *(-1.705 to 2.033)*	*0.011* *p=0.158* *(-0.004 to 0.027)*	*0.353** *p=0.579* *(-0.894 to 1.600)*	***1.107*** ***p=0.007*** ***(1.028 to 1.194)***	*1.057* *p=0.118* *(0.986 to 1.132)*	*1.219* *p=0.186* *(0.910 to 1.633)*	*1.019* *p=0.938* *(0.646 to 1.607)*
Smoking	*-0.736* *p=0.741* *(-5.091 to 3.619)*	*-0.637* *p=0.870* *(-8.291 to 7.016)*	*0.293** *p=0.829* *(-2.366 to 2.951)*	*-0.643** *p=0.668* *(-3.579 to 2.293)*	*-0.127** *p=0.891* *(-1.943 to 1.689)*	*0.944* *p=0.350* *(0.834 to 1.067)*	*1.076* *p=0.227* *(0.955 to 1.213)*	***1.644*** ***p=0.021*** ***(1.079 to 2.500)***	*1.327* *p=0.211* *(0.853 to 2.061)*
Body Mass Index	*0.425* *p=0.170* *(-0.181 to 1.031)*	*0.665* *p=0.098* *(-0.122 to 1.452)*	*0.012** *p=0.925* *(-0.235 to 0.259)*	*-0.203** *p=0.112* *(-0.460 to 0.055)*	*-0.195** *p=0.086* *(-0.418 to 0.028)*	*0.673** *p=0.529* *(0.196 to 2.312)*	*0.412** *p=0.051* *(0.169 to 1.005)*	*0.513** *p=0.756* *(0.008 to 34.674)*	*0.962* *p=0.085* *(0.920 to 1.005)*
Mean Arterial Pressure	*-0.114* *p=0.124* *(-0.259 to 0.031)*	*-0.007* *p=0.954* *(-0.243 to 0.229)*	***-0.069**** ***p=0.030*** ***(-0.130 to -0.007)***	*-0.001** *p=0.985* *(-0.056 to 0.055)*	*-0.018** *p=0.557* *(-0.077 to 0.042)*	*1.156** *p=0.368* *(0.841 to 1.589)*	*1.236** *p=0.156* *(0.923 to 1.660)*	*4.345** *p=0.059* *(0.944 to 20.045)*	*2.698** *p=0.181* *(0.630 to 11.535)*
Framingham Risk Score	***-0.166*** ***p=0.011*** ***(-0.295 to -0.038)***	*-0.116* *p=0.449* *(-0.417 to 0.185)*	*-0.050** *p=0.224* *(-0.131 to 0.031)*	*0.019** *p=0.544* *(-0.042 to 0.080)*	*0.024** *p=0.376* *(-0.030 to 0.078)*	*1.107** *p=0.587* *(0.767 to 1.592)*	***1.361**** ***p=0.030*** ***(1.030 to 1.803)***	***4.977**** ***p=0.042*** ***(1.062 to 23.335)***	*2.317** *p=0.327* *(0.432 to 12.445)*

Note that some values are reported as ratios due to a log transformation applied to some of the dependent variables. **Bold** values are significant at $p<0.05$. GEE, corrected for age and gender. Eye/brain parameters were chosen as the dependent variables. CRAE = Central Retinal Artery Equivalent, CRVE = Central Retinal Vein Equivalent, AVR = Arteriole-Venular Ratio, FDa/v = Fractial Dimension of Arteries/Veins, cTORTa/v = Curvature Tortuosity of Arteries/Veins, pWMH = Peritventricul White Matter Hyperintensities, dWMH = Deep White Matter Hyperintensities. * = in steps of 100, † = in steps of 10.

Supplementary table 2: Associations between retinal vascular parameters and white matter hyperintensities

	Periventricular white matter hyperintensities volume Ratio, p-value (95% CI of ratio)	**Deep white matter hyperintensities volume** Ratio, p-value (95% CI of ratio)
Central retinal artery equivalent*	*2.447, p=0.185 (0.652 to 9.204)*	*1.714, p=0.519 (0.333 to 8.790)*
Central retinal vein equivalent*	***3.148, p=0.015 (1.250 to 7.925)***	*2.009, p=0.244 (0.621 to 6.486)*
Arteriole-venular ratio	*0.174, p=0.204 (0.012 to 2.576)*	*0.292, p=0.476 (0.010 to 8.650)*
Fractal dimension of arteries	*1.380, p=0.833 (0.069 to 27.73)*	***306.902, p<0.001 (12.388 to 7603.263)***
Fractal dimension of veins	*3.126, p=0.522 (0.095 to 102.329)*	***30.761, p=0.042 (1.125 to 841.395)***
Curvature tortuosity of arteries†	*1.936, p=0.237 (0.648 to 5.792)*	*3.061, p=0.075 (0.891 to 10.510)*
Curvature tortuosity of veins†	*2.022, p=0.293 (0.544 to 7.508)*	***7.303, p=0.004 (1.853 to 28.787)***

Beta's are reported in ratios due to a log transformation applied to the dependent variables. **Bold** values are significant at $p<0.05$. GEE, corrected for age, gender, total intracranial volume and Framingham Risk Score. Brain parameters were chosen as the dependent variables. * reported in steps of 100, † reported in steps of 10^{-4}.